OBSERVATIONS ON *AMOEBA PROTEUS* VAR. *CYANEA* NOZAWA, ESPECIALLY ON ITS CRYSTAL INCLUSIONS AND NUCLEAR DIVISION

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ONE PLATE AND ELEVEN TEXT-FIGURES

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INTRODUCTION

I have been culturing, since September, 1937, a large amoeba, rather common in the vicinity of Kyoto. It resembles *Amoeba proteus* (Leidy), but may be distinguished from this by its somewhat smaller size, the bluish colour of its cytoplasm, and especially by the presence of two kinds of crystals. The name *A. cyanea** Nozawa was given to this apparently new amoeba in my preliminary report (1938). Upon observation, it has been revealed that the amoeba differs clearly in its crystal inclusions from *A. proteus*, commonly found in Europe and in America, although in its karyological features there is no essential distinction between the two. In this paper I propose to describe this amoeba somewhat in detail especially its crystal inclusions, nuclear structure and division.

Before going further I wish to express my sincere thanks to Prof. T. Komai for his generous guidance and encouragement throughout this work. My thanks are also due to Mr. K. Ono of this institute and to Mr. T. H. Abé of the Tokyo Imperial University, for their valuable advice.

MATERIAL AND METHODS

Observations were made on the original strain obtained at Kitasirakawa, Kyoto, as well as on the strains from Lake Biwa and other localities near Kyoto. Two rice grains added to 50 cc. of pond water in a Petri dish were supplied for food. The culture attains a good

* The generic name *Chaos* was used in my preliminary paper; the specific name should have been *cyaneum* instead of *cyaneus*.

condition in a thermostat adjusted at 20°C. in about two weeks. The dividing amoebae were isolated by means of a capillary pipette under a binocular microscope.

The crystal inclusions of both living and desiccated materials were examined. Total preparations were fixed and stained on slides. For fixation, Bouin's fluid, Carnoy's fluid with or without chloroform, alcohol-formalin with acetic acid (absolute alcohol 45 parts, formalin 10 parts, glacial acetic acid 2 parts), and acetic acid in various concentrations were employed. Delafield's hematoxylin and its modification with iron alum solution as in Heidenhain's method were used for staining. As the material is apt to be detached from the slide, alcoholic iron alum solution was preferable to the watery one (iron alum 1 gr., 70% alcohol 99 cc.). Feulgen's method, employed for the differentiation of the chromatin granules, gave good results. The material was hydrolyzed for 10 minutes at 70°C. The amoebae of the vegetative stage were embedded in paraffin and sectioned 5 micra in thickness. All the drawings were made by the aid of an Abbe camera.

CRYSTALS

In this section I shall describe the crystals from the toxonomic view point.

The four types of crystals. All the crystals belong to the rhombic system and may be classified under four different types.

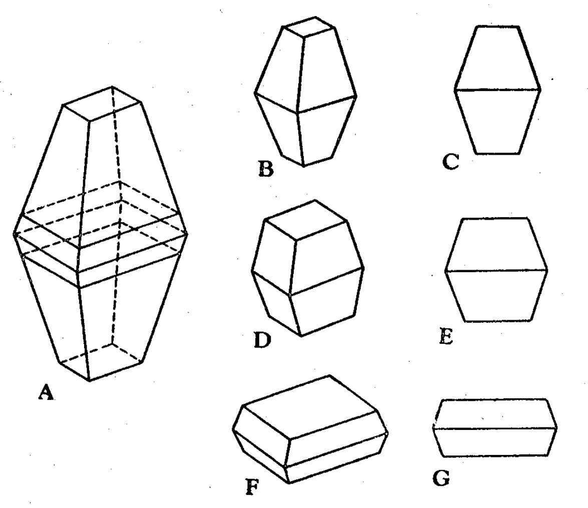

Fig. 1. Schematic figures showing the crystals of Type 1–3: A, illustrating truncation at the different parameter of the vertical axis to result in different types of crystals; B, C, Type 1; D, E, Type 3; F, G, Type 2.

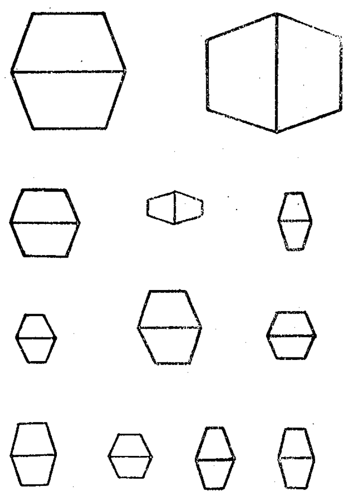

Fig. 2. Crystals of Type 1. ×1670.

Type 1 (Fig. 1, B, C; Fig. 2; Pl. 12, B), the commonest and found in all individuals, is a truncate bipyramidal crystal with a vertical axis longer than the other two axes. The vertical axis of the largest crystal measures 9.5 micra. In the lateral view the crystal, especially larger ones, is more depressed than that of *A. proteus s. s.* (cf. Mast and Doyle 1935). In vertical view the crystal always appears square.

Type 2 (Fig. 1, F, G; Fig. 3; Pl. 12, A, C, D, etc.), which occurs in 30–100% of the individuals, is a truncate bipyramidal crystal either square or oblong in vertical view whose vertical axis is shorter than the others. The longest edge is 9.0 micra in length; the vertical axis being more variable than the other axes, the thickness of the crystal varies extensively. In any case it is a bipyramidal crystal with truncate apices and is distinguished from Type 1 by its vertical axis which is the shortest. As far as I have been able to ascertain, this type of crystal is not recorded as being present in *A. proteus*, although it is common in *A. dubia* Sch., *Trichamoeba caerulea* Sch., and others.

Type 3 (Fig. 1, D, E; Pl. 12, F, I) is intermediate between Type 1 and Type 2 in form: the vertical axis is approximately equal in length to the edge, which measures anywhere up to 8.0 micra in the largest crystal. The type occurs in larger numbers than Type 2 but seems to be missing in *A. proteus*. These three types of crystals are connected by a series of intermediate forms.

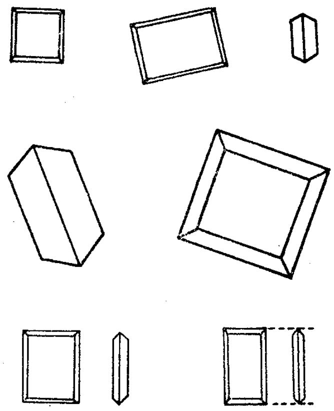

Fig. 3. Crystals of Type 2. ×1670.

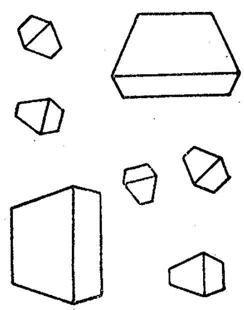

Fig. 4. Crystals of Type 4. ×1670.

Besides these types, there is another kind of crystal with an irregular shape, Type 4 (Fig. 4; Pl. 12, K, L). Each component pyramid is truncate at a different parameter of the vertical axis.

A few extremely thin crystals (attached to the refractive body) are

also found (Fig. 5). They differ from the plate-like crystals known in *A. proteus* and *A. dubia* because of their truncate bipyramidal form square or oblong in the vertical view, having a very short vertical axis and not being as irregular as in the plate-like ones. In the past two years, I came across several individuals which contained this type of crystal in abundance.

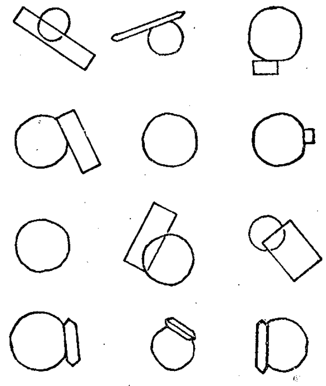

Fig. 5. Thin crystals of Type 2, attached to the refractive bodies. ×1670.

Distribution of crystals. Type 1 and Type 2 were first found in the strain from Kita-sirakawa. Subsequent examinations have revealed the presence of such crystals in the material from Lake Biwa (Table 1). They are also found in spherical individuals which have withdrawn all their pseudopodia.

Table 1. Distribution of crystals in a strain form Lake Biwa (Cult. No. 144). + indicates the quantity of crystals. Type 3 is included under Type 1.

Type 2 \ Type 1	+++	++	+
+++	—	2	1
++	10	8	—
+	21	—	—
—	28	—	—

To find the effect of culture media upon the crystals, amoebae were reared in the following: redistilled water, tap water, various pond water, 0.025% solution of Liebig's beef extract, and Chalkley solution (NaCl 0.1 gr., KCl 0.004 gr., CaCl 0.006 gr., redistilled water 1000 cc.). The same kind of crystals appeared in most of the individuals. This fact shows that the occurrence of these crystals is a specific characteristic of the present amoeba.

STRUCTURE OF THE RESTING NUCLEUS

Living nucleus and endosome. There is a well defined nuclear membrane and a layer of chromatin granules, about 2 micra in thickness, immediately under it (Fig. 6). Careful examination with an oil-immersion lens shows that the central chromatin granules are universely distributed throughout the entire nucleus. All the chromatin granules disappear in specimens pressed under a cover slip, probably due to the

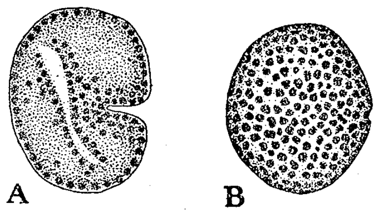

Fig. 6. Nucleus of a living amoeba; ×600. A, optical cross-section of a nucleus with marked invagination; B, surface view of a nucleus.

absorption of water in the nucleus. Although hundreds of living individuals were examined, no endosome was found.

The effect of fixatives on the living nucleus was studied with the following results:—

The nuclear membrane and the underlying granules are fixed without any perceptible change by 1–5% acetic acid. The chromatin granules which are hardly visible in the living condition form a large central mass around which a more or less distinct hyaline area is formed (Fig. 7, A). This chromatin mass greatly reminds one of the "endosome" or "karyosome" described by previous investigators. It is no doubt a kind of artifact produced by acetic acid, and found in many of the total preparations (Fig. 8).

In 10–30% acetic acid, the nucleus swells and the nuclear membrane becomes detached from the layer of chromatin granules, leaving a more or less clear hyaline area. The fine chromatin granules aggregate in the central part of the nucleus forming a reticulate structure (Fig. 7, B).

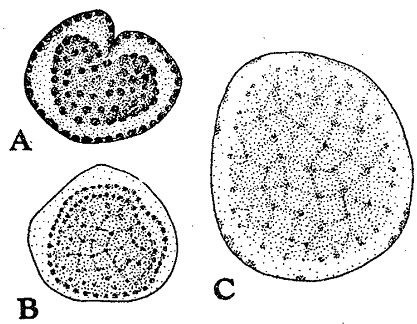

Fig. 7. Artifact produced in the nucleus. A, treated with 1% acetic acid, ×600; B, treated with 25% acetic acid, ×400; C, treated with 50% acetic acid, ×600.

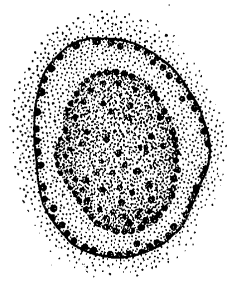

Fig. 8. Endosome-like artifact produced by Carnoy's fluid, optical cross-section, ×900.

Acetic acid stronger than 30% produces a swelling of the whole nucleus and the appearance of a hyaline area between the nuclear membrane and the finely granulated chromatin mass. A marked reticulation appears. The mass of central chromatin never takes the form of an "endosome" (Fig. 7, C). Thus the various kinds of artifacts are induced in the nucleus by acetic acid of different concentrations.

STAINED NUCLEUS AND TWO KINDS OF CHROMATIN GRANULES

Both the sectioned and total preparations reveal that the chromatin granules are arranged in a distinct layer immediately under the nuclear membrane. But as clearly shown by observations stated above, the central granules are markedly changed by fixation. In a well preserved specimen, chromatin granules finer than the peripheral ones are seen in all parts of the nucleus without producing any definite structure like an endosome. No hyaline area seems to exist between the peripheral and central granules in the living nucleus.

Feulgen's method reveals the difference between the two kinds of granules as pointed out by Chalkley (1936); the peripheral granules do not stain, while central granules become reddish violet. By Delafield's hematoxylin the two kinds of chromatin in hydrolyzed preparations are stained in the same tone (Fig. 9). The reticulate structure is stained by Feulgen's method if the material is fixed with concentrated acetic acid or desiccated on a slide. These results suggest that the reticulation is an artifact produced by fixation.

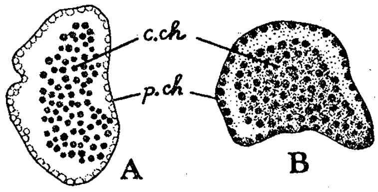

Fig. 9. Two kinds of chromatin granules, ×900. A, nucleus stained by Feulgen's method; B, nucleus stained by Delafield's hematoxylin after hydrolysis (control). *c. ch.*, central chromatin granules positive to Feulgen reaction; *p. ch.*, peripheral chromatin granules negative to this test. Sectioned preparations.

CHANGE OF BODY FORM DURING DIVISION

The changes which take place in this species during division are similar to those in *A. proteus* and *A. dubia* (Fig. 10). Prior to division, the amoeba withdraws all its long pseudopodia and assumes the shape of the "division sphere". Short pseudopodia in the form of swellings are left. The "division sphere" lasts from the prophase to the late anaphase. In the telophase the body becomes irregular in shape and produces some long pseudopodia. No long cytoplasmic connection, like that which appears between the daughter individuals in *A. dubia*, is formed.

NUCLEAR CHANGE DURING DIVISION

As the dividing amoeba is not transparent, the nuclear change may be observed only after fixation and staining.

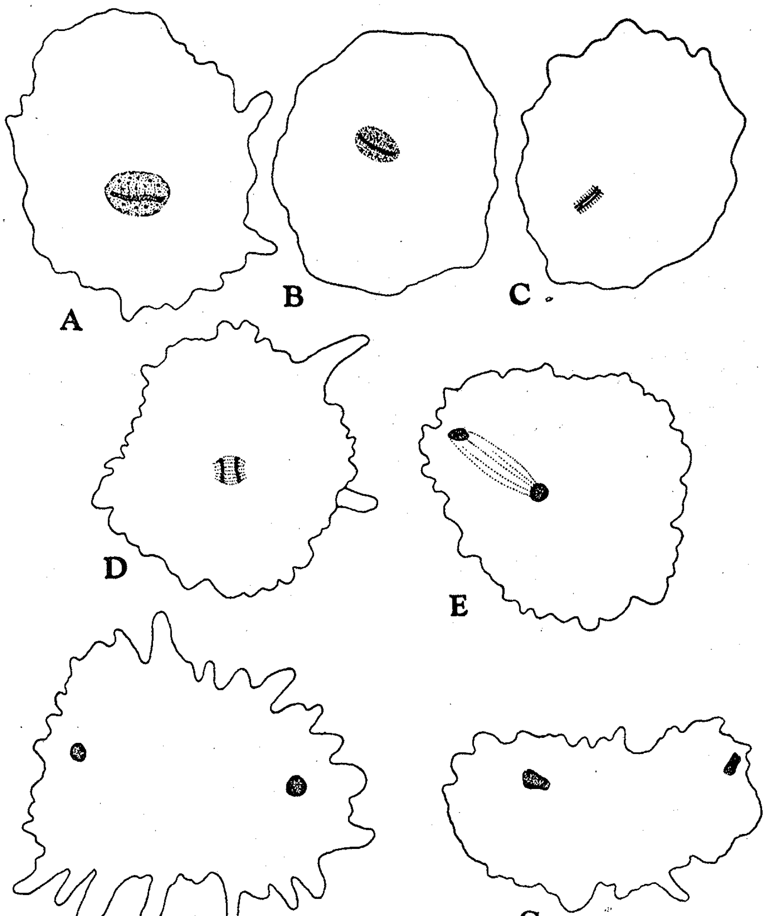

Fig. 10. Relation between the form of the body and the phases of nuclear division, ×190. A, B, prophase; C, metaphase; D, anaphase; E, early telophase; F, G, telophase. Total permanent preparations.

Prophase. The nucleus swells somewhat at prophase and the nuclear membrane becomes more or less obscure. Chromatin granules aggregate in a plane parallel to the longer axis of the nucleus. The number of peripheral granules decreases; the chromatin granules all stain faintly (Fig. 11, B). By late prophase, the nucleus becomes barrel-shaped and the nuclear membrane still more obscure. Even when the equatorial plate is nearly completed, some of the peripheral granules still remain without any change, especially near the poles of the shorter axis (Fig. 11, C). It could not be ascertained whether or not these granules participate in the formation of the equatorial plate in more advanced stages. Distinct spindle fibers, hundreds in number, strech between the plate and nuclear membrane.

Metaphase. The equatorial plate is already completed at this

stage. Distinct chromosomes are found especially in the periphery of the plate. They are so small and numerous that it is hardly possible to count them—there are probably hundreds of chromosomes. In this period the nuclear membrane is almost invisible. The distinction of

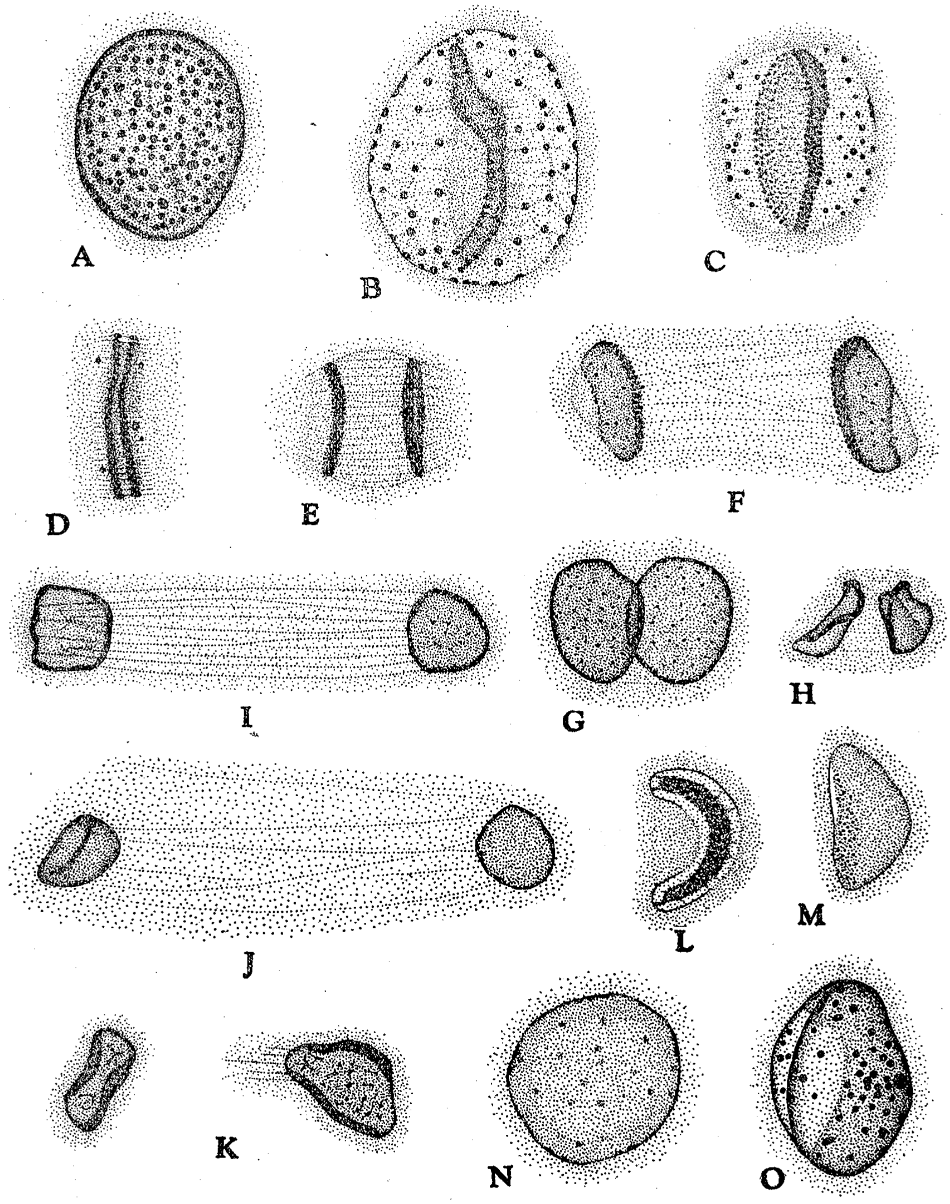

Fig. 11. Successive stages of nuclear division, all ×900. A, resting nucleus; B, early prophase; C, late prophase; D, metaphase; chromosome plate just separated; E, anaphase; F, late anaphase; G, late anaphase in polar view; H, late anaphase, abnormal figure; I—N, telophase; K, curved daughter nuclei; L, M, side views; N, appearance of central and peripheral granules; O, daughter nucleus one hour after seperation of cell body.

the karyoplasm from the surrounding cytoplasm is quite obscure. A few peripheral granules still remain. On one slide some chromosomes were observed which had apparently just separated from their mate, for they still retained the spindle fibers extending between each pair, thus indicating the division of each chromosome (Fig. 11, D).

Anaphase. The components of the equatorial plate or chromosome plate are now divided *en masse*, in two, and move towards each pole. The plate appears concave. Though the chromosomes in the peripheral part of the plate still stain deeply, those in the central part are faint and appear rather homogeneous. The spindle fibers near the poles as well as between the plates are fully formed (Fig. 11, E). In the late anaphase, the spindle fibers are distinct between the plates and become poleplasms, having a parachute-like appearance (Fig. 11, F).

Telophase. In the stages from metaphase to early telophase, the chromosome plate or daughter nucleus is reduced in diameter (Fig. 11, D—J). The daughter nucleus of the early telophase consists of a homogeneous central part and a thin and deeply stained margin. The spindle fibers between the daughter nuclei are greatly elongated while the poleplasm becomes indistinct (Fig. 11, G—J). In the mid telophase, the daughter nucleus curves and swells (Fig. 11, K, L). The spindle fibers are absorbed and a thin nuclear membrane appears (Fig. 11, L, M). The cell body divides either at this time or in a little more advanced stage (Fig. 10, G). The daughter nucleus increases gradually in size and becomes homogeneous. Chromatin granules appear immediately both under the membrane and in the central part of the homogeneous nucleus (Fig. 11, N). The former are the origin of the peripheral granules, while the latter become the central granules of the next generation. In one hour after the division of the cell body, a large quantity of the peripheral and central granules appear in the nucleus (Fig. 11, O). Thus the reorganization of the daughter nucleus takes place within a few hours after the cell division.

DISCUSSION

Crystals. The crystals in *A. proteus* (Leidy) *s. s.* have been drawn and described by many investigators; they are the following:

Truncate bipyramidal crystal: Leidy (1879), Penard (1902), Schubotz (1905), Prandtl (1907), Vonwiller (1913, 1918), Schaeffer (1917), Mast (1926), Mast and Doyle (1935, 1935a).

Pointed bipyramidal crystal: Greef (1891), Penard (1902), Vonwiller (1913, 1918).

Pointed semi-bipyramidal crystal: Vonwiller (1918).

Plate-like crystal: Schubotz (1905), Mast (1926), Mast and Doyle (1935, 1935a).

These different kinds of crystals can all be classified under two major types: a truncate bipyramidal crystal with a vertical axis longer than the other axes, and a plate-like crystal (of Mast and Doyle) in which the vertical axis is shorter than the others and more or less irregular in shape. From the figures of the previous investigators, it is clear that the pointed bipyramidal crystal as well as its semi-bipyramidal derivative is a kind of plate-like crystal.

Here a question arises concerning the type of crystals found in *A. proteus s. s.* None of the authors cited above have drawn or recorded a thin truncate bipyramidal crystal having a vertical axis shorter than the other two axes such as found in *A. dubia* and also in the present amoeba. According to Schaeffer (1917), one of the distinctive features between *A. proteus* and *A. dubia* is the fact that the former is provided only with the crystal of my Type 1, while the latter has both my Type 1 and Type 2. Even such extensive works as Vonwiller's, and Mast and Doyle's do not contain any record of the Type 2 crystal in *A. proteus*. It is highly improbable that the presence of this type of crystal has been overlooked. In the present amoeba, on the other hand, Type 1, Type 2 and the intermediate Type 3 appear in 30–100% of the individuals collected from the various places mentioned.

No plate-like crystal, as that recorded to be in *A. proteus s. s.* has been found in the present amoeba. Even those extremely thin crystals attached to the refractive body, revealed by crucial examination, were not a quadrate but a truncate bipyramidal type with a very short vertical axis. The irregular crystal, Type 4, is commonly found mixed in other types in this amoeba.

It seems important that the same kinds of crystals appear in amoebae raised in various kinds of culture media, as for instance, in those of both redistilled water and Chalkley solution. Thus the present amoeba is definitely different from *A. poteus s. s.*, as much as *A. dubia* is from *A. proteus*, with respect to crystal inclusions.

Karyological features. According to Dawson, Kessler and Silverstein (1937), a fixed and stained preparation of *A. proteus* shows " the central portion of the nucleus to be composed of a lightly staining

fairly homogeneous mass of finely granular material" (p. 128). The present amoeba shows the same feature. Peripheral granules are arranged in a disticnt layer just under the nuclear membrane. The nucleus contains homogeneous nuclear sap in which central granules are evently scattered.*

The "endosome" or "karyosome" of *A. proteus* is described by Gruber (1912), Doflein (1918) and Taylor (1923–1928), and that of *A. discoides* Sch. by Hayes (1938). Bělař (1926), and Dawson and his co-workers (1937) are of the opinion that the clearly defined "endosome" is probably an artifact produced by fixatives. As stated above, an endosome-like structure may be produced by acetic acid of various concentrations. Thus it is highly probable that the "endosome", in large amoebae, is a substance artificially produced by fixation.

Microchemical differences among the chromatin granules in *A. proteus* have been confirmed by Chalkley (1936). Practically the same dissimilarities were found by Reichenow (1928) in an amoeba of the *Verrucosa* type. In both the total and sectioned preparations of the present amoeba, the peripheral granules are negative to Feulgen reaction but the central granules stain reddish violet. The preparations treated by this method, however, show a smaller quantity of chromatin substance in the center of the nucleus than those stained by hematoxylin (Fig. 9). This may be due to the fact that the nuclear sap is negative to Feulgen reaction, but is stained lightly by hematoxylin. Dawson and his co-workers state: "by this (Feulgen's) method both the peripheral granules and the central portion stained, the former being somewhat more intense in its reaction" (p. 128). It is clear, however, from both Chalkley's and my results that this reverse staining reaction was brought about by some faulty technique. The peripheral granules resemble the "endosome" of little amoebae in lacking the nucleic acid, though a more minute comparative study is needed to elucidate this point.

In the present amoeba the nuclear division is typically promitotic and is closely allied to the process in *A. proteus s. s.* But there are important disagreements between the statements of Chalkley and Dawson even with this same *proteus*. Thus it is hard to decide whether the apparent differences between *A. proteus* and the present

* The term "endosome" was used by Dawson and his co-workers (1937) for the fairly homogeneous internal part of the nucleus, but I shall avoid the term, because to my mind it seems rather ambiguous.

amoeba in the nuclear division, is real or not, since our knowledge of nuclear division in large amoebae is still very deficient. The nuclear division of large amoebae seems to be less differentiated and of smaller taxonomic value than in little amoebae such as *Vahlkampfia* and *Hartmanella*.

The presence or absence of a nuclear membrane during karyokinesis has been discussed by some authors. Chalkley is of the opinion that it disappears in the metaphase of *A. proteus*, while Dawson and his co-workers maintain that the membrane persists throughout the process. It is clear, however, after tracing the nuclear membrane of the present amoeba in the total preparation to the late prophase, that the membrane is much thinner in the karyokinesis stage than in the vegetative condition; it is possible that the surface of contact of the cytoplasm and karyoplasm gives an appearance of a thin membrane because of the difference in viscosity, optical index, and affinity to dyes between the two. This observation agrees with Chalkley's on his total preparation of *A. proteus*, and seems to show that the new membrane is formed after the late anaphase.

Specific name of the present amoeba. As stated above, the present amoeba differs in its crystal inclusions from *A. proteus s. s.* found in Europe and America. This difference corresponds to that found between *A. dubia* and *A. proteus*. The present amoeba may be considered as intermediate between these two common large amoebae. In the general karyological features, on the other hand, the present amoeba is allied to *A. proteus s. s.* Thus it seems best to regard this amoeba as not an independent species, but as a variety of *A. proteus* (Leidy) *s. s.* for the present. Hence the name will be *Amoeba proteus* var. *cyanea* Nozawa.

SUMMARY

1. A large amoeba commonly found in the vicinity of Kyoto is described in detail, with emphasis on its crystal inclusions, nuclear structure and division. The findings are compared with statements by previous authors on *Amoeba proteus* (Leidy) *s. s.*

2. Four types of crystal inclusions were found: 1) a truncate bipyramidal crystal with a vertical axis longer than the other two, 2) the same, but having a vertical axis shortest of all, 3) an intermediate form between the former two types, and 4) a bipyramidal crystal, each component of which is truncate at a different parameter of the vertical axis.

3. These four kinds of crystals are found in the individuals of different locarities and of different culture media, so that their occurrence seems to be a characteristic fracture of the present amoeba.

4. There is no essential difference in the karyological features between *A. proteus s. s.* and the present amoeba. Feulgen's method reveals a microchemical distinction existing between the peripheral and central chromatin, the former being negative to this test, while the latter is stained reddish violet.

5. The peripheral granules are visible in living specimens; they are arranged directly under the nuclear membrane forming a regular layer. The central granules are evenly distributed in the nuclear sap; they are more minute than the peripheral granules and hardly visible in the living specimen.

6. Various artifacts are produced by some fixatives in the otherwise fairly homogeneous looking nucleus. The "endosome" of large amoebae, reported by previous authors seems to be nothing but an aggregation of the central granules brought about by fixatives.

7. The nuclear division is a kind of promitosis. The chromosomes are so minute and numerous that it is hardly possible to give their number accurately—these are probably hundreds.

8. The nuclear membrane seems to disappear in the metaphase and reappear in the early telophase. No centrosome is found throughout the process, though distinct spindle fibers are formed.

9. It seems best to regard the present large amoeba as *Amoeba proteus* var. *cyanea* Nozawa, a variety of *A. proteus* (Leidy) *s. s.*

POSTSCRIPT

After this manuscript had been prepared, I found W. Liesche's "Die Kern- und Fortpflanzungsverhältnisse von *Amoeba proteus* (Pall.)" published in Archiv für Protistenkunde **91**: 135–186 (1938). His results on the nuclear structure and division seem to agree for the most part with my findings here recorded except for the multipolarity of spindle fibers (Ber. ü. d. wiss. Biol., **49**: 197–198).

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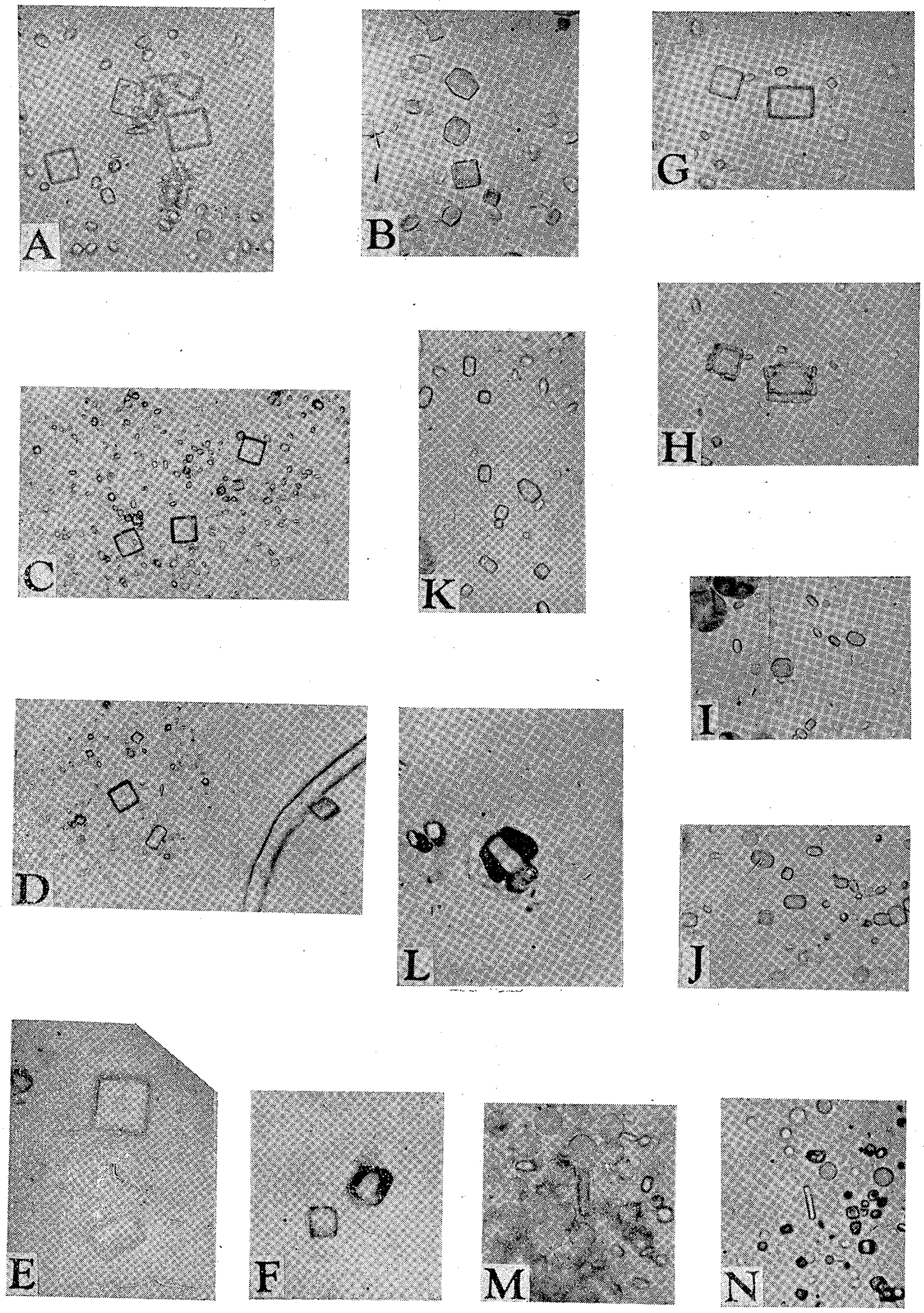
EXPLANATION OF PLATE 12

A. Crystals of Type 2, vertical view.
B. Crystals of Type 1, lateral view.
C. Crystals of Type 2, vertical view.
D. The same, showing the lateral view.
E. The same highly magnified.
F. A crystal of Type 3.
G. Crystals of Type 2, vertical view.
H. The same, crushed under a cover slip.
I. A crystal of Type 3, lateral view.
J. A crystal of Type 2, lateral view.
K. A crystal of Type 4, lateral view.
L. A crystal of Type 4, lateral view.
M. A crystal of Type 2, showing the edge in the lateral view.
N. A crystal of Type 2, lateral view.

Magnification: C, D, ×460; others, ×740.



K. NOZAWA: OBSERVATIONS ON *AMOEBA PROTEUS* VAR. *CYANEA* NOZAWA